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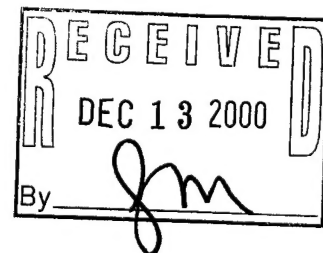
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**TWENTY-FIRST AMINOACYL-tRNA SYNTHETASE-SUPPRESSOR tRNA PAIRS
FOR POSSIBLE USE IN SITE-SPECIFIC INCORPORATION OF AMINO ACID
ANALOGUES INTO PROTEINS IN EUKARYOTES AND IN EUBACTERIA**

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ABSTRACT

Two critical requirements for developing methods for the site-specific incorporation of amino acid analogues into proteins *in vivo* are (i) a suppressor tRNA that is not aminoacylated by any of the endogenous aminoacyl-tRNA synthetases (aaRSs) and (ii) an aminoacyl-tRNA synthetase, which aminoacylates the suppressor tRNA but no other tRNA in the cell. Here, we describe two such aaRS/suppressor tRNA pairs, one for use in the yeast *S. cerevisiae*, and another for use in *E. coli*. The “21st synthetase/tRNA pairs” include *E. coli* glutaminyl-tRNA synthetase (GlnRS) along with an amber suppressor derived from human initiator tRNA, for use in yeast, and mutants of the yeast tyrosyl-tRNA synthetase (TyrRS) along with an amber suppressor derived from *E. coli* initiator tRNA, for use in *E. coli*. The suppressor tRNAs are aminoacylated *in vivo* only in the presence of the heterologous aaRSs and the aminoacylated tRNAs function efficiently in suppression of amber codons. Plasmids carrying the *E. coli* GlnRS gene can be stably maintained in yeast. However, plasmids carrying the yeast TyrRS gene could not be stably maintained in *E. coli*. This is most likely because the wild type yeast TyrRS misaminoacylates the *E. coli* proline tRNA. Using error-prone PCR, we have isolated and characterized three mutants of yeast TyrRS, which can be stably expressed in *E. coli*. These mutants still aminoacylate the suppressor tRNA essentially quantitatively *in vivo* but show increased discrimination *in vitro* for the suppressor tRNA over the *E. coli* proline tRNA by factors of 2.2 to 6.8 fold.

INTRODUCTION

The site-specific incorporation of amino acid analogues into proteins *in vitro* has been used successfully for a number of applications and these experiments have demonstrated the wide range of amino acid analogues, which are accepted by the translational machinery (1-4). The most common approach involves the read through of an amber stop codon by an amber suppressor tRNA, which is chemically aminoacylated with the analogue of choice. Unfortunately, the procedure in general provides only small amounts of protein and use of semi-synthetic methods for the preparation of the aminoacyl-tRNA has limited its general application. Recent improvements to the system include increasing the overall translational efficiency or increasing the suppression-to-termination ratio by the use of extracts containing temperature sensitive release factors (5-7). Isotopically-labeled amino acids and amino acid analogues such as fluorotyrosine which are recognized by one of the aminoacyl-tRNA synthetases (aaRSs) can also be incorporated site-specifically into proteins using a separate enzymatic aminoacylation step, thus avoiding the need for chemical aminoacylation (8, 9). Microinjection of suppressor tRNAs aminoacylated with amino acid analogues into *Xenopus* oocytes has allowed the *in vivo* structure-function analysis of ion channel proteins (10), but extremely low protein yields limit further work.

The availability of methods to incorporate amino acid analogues site-specifically into proteins *in vivo* in eukaryotes and in eubacteria would greatly expand the scope and utility of unnatural amino acid mutagenesis. First, there is the potential to synthesize large amounts of the protein, making this a particularly useful technique for preparing material for spectroscopic and crystallographic analyses. Second, potential problems associated with post-translational modifications and folding may be overcome for some proteins if a eukaryotic system is used.

And finally, the availability of an *in vivo* system opens the door to the study of *in vivo* structure-function studies including studies of protein-protein interactions, protein localization, etc., using amino acid analogues that carry photoactivatable groups, fluorescent groups and other chemically reactive groups.

The basic approach for the *in vivo* work relies on a suppressor tRNA aminoacylated with an amino acid analogue by a mutant aminoacyl-tRNA synthetase (aaRS), to insert the analogue at a specific site in a protein (11). The site of insertion of the analogue is specified by an appropriately placed stop codon within the gene for the protein of interest. This approach has two key requirements: (i) a suppressor tRNA, which cannot be aminoacylated by any of the endogenous aaRSs in a cell and (ii) an aaRS, which aminoacylates the suppressor tRNA but no other tRNA in the cell. Therefore, an important first goal is to identify such an aaRS/suppressor tRNA pair (21st synthetase/tRNA pair). Once such a 21st synthetase/tRNA pair is identified, the next goal is to isolate mutants of the aaRS which will attach the desired amino acid analogue, but not the normal amino acid, onto the suppressor tRNA.

In this paper, we describe two candidate 21st synthetase/tRNA pairs, one for use in yeast and another for use in *E. coli*. The yeast system represents the first 21st synthetase/tRNA pair for use in a eukaryote. The suppressor tRNAs (12, 13) are either derived from a tRNA of another eukaryotic organism (human initiator tRNA for use in yeast) or from a tRNA of the same organism (*E. coli* initiator tRNA₂^{fMet} for use in *E. coli*). The 21st synthetases are *E. coli* GlnRS for use in yeast and mutants of yeast TyrRS for use in *E. coli*. The 21st synthetase/tRNA pairs that we have developed are different in principle from the ones described by Schultz and coworkers for use in *E. coli*, which are based on import of both the aaRS and the suppressor derived from a cognate tRNA from a heterologous organism (14, 15).

MATERIALS AND METHODS

General. Standard *E. coli* and yeast genetic techniques were used (16, 17). Yeast cells were transformed using the method of Tan *et al.* (18). Synthetic minimal media for growth and maintenance of yeast cells were supplemented with 2% raffinose + 2% glucose or 2% raffinose + 2% galactose, and amino acids to give SRD or SRG, respectively. Radiolabelled amino acids were from New England Nuclear, oligonucleotides were from Genosys Biotechnologies, anti-myc antibody was from Boehringer-Mannheim and anti-tetra-His antibody was from Qiagen.

Strains. *Escherichia coli* strains DH5 α and JM109 were used for plasmid propagation and isolation. *E. coli* CA274 [*lacZ*125(*am*) *trp*49(*am*) *relA*1 *spoT*1] and the isogenic Su⁺ strain CA275 (CA274 *supF*) were used for *in vivo* screening of stably expressed *Saccharomyces cerevisiae* TyrRS mutants. *S. cerevisiae* HEY301-129 [*a met*8-1*am trp*1-1*am his*4-580*am leu*2-3,112 *ura*3-1 *ade*1 *can*1-100] (19) was used as a tester strain for suppression in yeast.

Plasmids. The plasmids pETTyrRS, pACamG, and pRSVCATam27 carrying the U2:A71/U35A36/G72 mutant tRNA₂^{Met} gene have been described previously (20). *E. coli* UQ27 containing the plasmid pUCProRS was obtained from W. McClain. The plasmid pETTyrRS-myc was constructed by replacement of the *Stu*I-*Bam*HI fragment of pETTyrRS with a PCR-generated fragment comprising the same 3' sequence of the gene with the addition of bases coding for the eleven amino acid c-myc tag. For overexpression, the three mutant TyrRS genes were cloned into pET3d (Novagen). The plasmid pUC8pro1, containing the *E. coli* tRNA₁^{Pro} gene was constructed by PCR amplification of the *proK* gene, including the promoter and termination sequences, from *E. coli* genomic DNA, and subsequent cloning into the *Sma*I site of pUC8.

pScM, a YEp420 derived 2 μ *URA*⁺ plasmid contains the wild type human initiator tRNA coding sequence downstream of the yeast tRNA^{Arg} 5'-flanking sequence (21). The wild type initiator tRNA coding sequence was replaced by a fragment carrying the U35A36/U50G51:C63A64/U54/C60 mutations in the tRNA (Figure 1, left) to yield the plasmid pScM.M2. For inducible expression of *E. coli* GlnRS in yeast, a c-His6 tagged version of the *E. coli* GlnRS gene was cloned under the control of the GAL1 promoter of the 2 μ *LEU*⁺ plasmid pESC-LEU (Stratagene). The pESC-LEU.GlnRS_{His6} was constructed by amplifying the gene encoding the c-His6 tagged *E. coli* GlnRS from pQE16-GlnRS (22) and inserting a sequence of seven A residues directly upstream of the initiation codon ATG by using PCR. The PCR product was digested with *Bam*HI and *Hind*III and cloned into the multiple cloning site of pESC-LEU.

***In vivo* selection and characterization of stably-expressed *S. cerevisiae* TyrRS mutants.** A library of c-myc tagged mutant yeast TyrRS genes was prepared by error-prone PCR (23) using pETTyrRS-myc as a template and cloned between the *Xma*I and *Pst*I sites of the IPTG inducible expression vector pKK223-3 (Pharmacia). The mixture of plasmids was electroporated into competent CA274 cells carrying the pACamG plasmid and chloramphenicol resistant (Cm^r) colonies were selected on plates containing carbenicillin (50 μ g/mL), tetracycline (10 μ g/mL), and chloramphenicol (100 μ g/mL) at 37°. The Su⁺ phenotype of the Cm^r colonies was confirmed by scoring for growth on M9 minimal plates containing 0.4% lactose and by colony color on MacConkey plates containing carbenicillin, tetracycline and lactose. Colonies were inoculated into 2x YT containing all three antibiotics, grown to midlog phase, and induced with 1 mM IPTG. After an additional four hours of growth, total tRNAs were isolated and the extent of aminoacylation of tRNA was determined as described below.

Purification of wild type and mutant *S. cerevisiae* TyrRS. The wild type, myc-tagged and three mutant TyrRS proteins were overproduced in *E. coli* NovaBlue (DE3) and purified by modification of a previously described method (20). Fractions containing TyrRS were pooled and concentrated using an Amicon 30 spin filter, then dialyzed against storage buffer (50 mM potassium phosphate, pH 7.2; 5 mM DTT; 150 mM KCl; 50% glycerol) and stored at -20°C.

Enzyme assays. The incubation mixture for aminoacylation contained 30 mM HEPES.KOH, pH 7.5; 50 mM KCl; 8 mM MgCl₂; 2 mM DTT; 3 mM ATP; 15 μM ³H-tyrosine (specific activity 20 - 33 μCi/nmol); 0.18 mg/mL BSA; tRNA and TyrRS. The enzyme was diluted in a solution containing 15 mM potassium phosphate, pH 7.5; 100 mM KCl; 4 mM DTT; 10% glycerol; and 0.18 mg/mL BSA. Kinetic parameters were determined by Lineweaver-Burk analysis. The rate of aminoacylation was linear over the enzyme concentrations used and the extent of tRNA aminoacylation was limited to less than 10%.

Purification of tRNAs. Yeast tRNA^{Tyr} was purified from an enriched mixture of yeast tRNA^{Tyr} and tRNA^{Phe} from counter current distribution of total yeast tRNA (24) by electrophoresis on 12% nondenaturing polyacrylamide gels. The suppressor tRNA derived from *E. coli* tRNA₂^{fMet} and *E. coli* tRNA^{Pro} were isolated from *E. coli* B105 cells carrying the pRSVCATam27 (U2:A71/U35A36/G72) plasmid and *E. coli* JM109 carrying the pUC8pro1 plasmid, respectively, by phenol extraction and subsequently purified by electrophoresis on 12% nondenaturing polyacrylamide gels (25). Purity of the tRNAs was determined to be greater than 90% by aminoacylation with an appropriate aaRS preparation in all cases.

Extent of aminoacylation of suppressor tRNAs *in vivo*. Total tRNA from *E. coli* was isolated under acidic conditions using the guanidine thiocyanate-phenol-chloroform method (26) (Tri-Reagent; Molecular Research Center, Inc.). Total tRNA from yeast was isolated as described

(27). The extent of aminoacylation was determined by fractionation of the tRNAs by acid-urea gel electrophoresis, followed by detection of the suppressor tRNAs by Northern blot hybridization with specific oligonucleotides probes (28).

RESULTS

21st Synthetase/tRNA pair for use in yeast.

We reported previously on a mutant human initiator tRNA that can act as an amber suppressor in mammalian COS1 cells (13). This tRNA has the U35A36 mutations in the anticodon sequence, which allows it to read the UAG codon and the U50G51:C63A64 mutations in the TΨC stem, which allows it to participate in elongation. This mutant tRNA is not aminoacylated by any of the mammalian aaRSs, but is aminoacylated by *E. coli* GlnRS and, therefore, acts as an amber suppressor in mammalian cells expressing *E. coli* GlnRS. The mutant tRNA to be used as part of the 21st synthetase/tRNA pair in yeast has additional mutations, U54 and C60 in the TΨC loop (Figure 1, left), designed to make the tRNA more active in elongation (29).

The gene encoding the human initiator tRNA is not transcribed by yeast RNA polymerase III in the context of its own 5'-flanking sequence (30). Therefore, for expression in yeast, the mutant human initiator tRNA coding sequence was cloned downstream of the 5'-flanking sequence of the yeast tRNA^{Arg} gene (21). This construct (pScM.M2) expresses the mutant human initiator tRNA constitutively. To express the *E. coli* GlnRS in yeast, we used plasmid pESC-LEU.GlnRS_{His6}, which carries the *E. coli* GlnRS gene appropriately modified for expression in yeast (see Materials and Methods), under the control of the inducible yeast GAL1 promoter.

The mutant human initiator tRNA is aminoacylated only in yeast cells expressing the *E. coli* GlnRS. Transformants of *S. cerevisiae* HEY301-129 harboring pESC-LEU.GlnRS_{His6}/pScM.M2 were grown in selective medium in the absence or presence of galactose. Immunoblot analysis showed that *E. coli* GlnRS was produced only in cells grown in presence of galactose (data not shown). tRNAs were isolated from cells under acidic conditions and the extent of *in vivo* aminoacylation of tRNA was determined by acid-urea gel electrophoresis followed by Northern blot analysis with a probe specific for the M2 mutant tRNA (28). Aminoacylation of the mutant tRNA in yeast is strictly dependent on expression of *E. coli* GlnRS (Figure 2A). PhosphorImager analysis of the blot showed that approximately 45% of the tRNA was aminoacylated.

The mutant human initiator tRNA acts as a suppressor of amber codons only in yeast cells expressing *E. coli* GlnRS. *S. cerevisiae* HEY301-129 has three suppressible amber alleles (*met8-1*, *trp1-1*, *his4-580*) (19). To test for tRNA-mediated suppression of these amber alleles, HEY301-129 cells harboring pESC-LEU.GlnRS_{His6} or the pESC-LEU plasmid without the GlnRS gene were transformed with pScM.M2. *URA*⁺*LEU*⁺ transformants were isolated and streaked on selective minimal medium plates supplemented with two of the three amino acids. Suppression is indicated by cell growth in the absence of tryptophan, histidine, or methionine. Figure 2B shows the results obtained for the methionine marker. Expression of the mutant initiator tRNA alone does not result in suppression of the *met8-1* allele, however, co-expression of *E. coli* GlnRS along with the mutant initiator tRNA results in suppression. Suppression of the *his4-580* allele was also strictly dependent on the co-expression of *E. coli* GlnRS_{His6} and the mutant tRNA (data not shown). Control experiments showed that co-expression of the wild type human initiator tRNA and *E. coli* GlnRS did not result in suppression of the *met8-1* or the *his4-*

580 amber alleles. All transformants grew in the absence of tryptophan indicating suppressor-tRNA independent readthrough of the *trp1-1* allele, which is known to be a leaky mutation (19).

We also compared the growth rates of HEY301-129 carrying the 21st synthetase/tRNA pair, grown in the SRG minimal medium lacking either methionine or histidine, to that of transformants grown in the presence of methionine, histidine, and tryptophan (complete medium). After an initial lag phase, the doubling times of cells grown in medium lacking methionine or histidine was 360 and 375 min, respectively, compared to the doubling time of 255 min of cells grown in complete medium. These results suggest that although only 45% of the suppressor tRNA is aminoacylated in HEY301-129 (Figure 2A), the suppressor activity of the tRNA is high enough to produce adequate amounts of the methionine and histidine biosynthesis enzymes for good growth in medium lacking one or the other of these amino acids.

21st Synthetase/tRNA pair for use in *E. coli*.

In the course of studies on *E. coli* initiator tRNA₂^{fMet} designed to identify elements in the tRNA important for its function, we previously isolated a mutant tRNA (U35A36/G72 mutant) that is a suppressor of amber codons in *E. coli* (31). This tRNA is aminoacylated by *E. coli* GlnRS. Introduction of further mutations (U2:A71) resulted in a tRNA (Figure 1, right) that is now an extremely poor amber suppressor in *E. coli*, because it is not aminoacylated by any of the other aaRSs in *E. coli* (20). *In vitro* also, this tRNA is not aminoacylated by purified preparations of *E. coli* GlnRS, LysRS, MetRS, TyrRS or a highly enriched preparation of ProRS (Kowal and RajBhandary, unpublished observations). The mutant tRNA contains the C1:G72 base pair, which is one of the critical determinants for yeast TyrRS recognition (12, 32). Because of this, it is aminoacylated by yeast TyrRS and is, therefore, active as an amber suppressor in yeast (12). Based on this observation, we used a genetic selection system requiring the activity of the

suppressor tRNA in *E. coli* to clone the gene for yeast TyrRS in a low copy yeast shuttle vector (20). If the yeast TyrRS does not aminoacylate any of the endogenous *E. coli* tRNAs, the yeast TyrRS and the U2:A71/U35A36/G72 mutant *E. coli* tRNA₂^{fMet} (Figure 1, right) would comprise a potential 21st synthetase/tRNA pair for use in *E. coli*.

Wild type yeast TyrRS can aminoacylate *E. coli* tRNA^{Pro}. Our initial attempts to clone and express the yeast TyrRS in *E. coli* using the standard expression vector pKK223-3 yielded a lethal phenotype, which could only partially be overcome by growth of the cells in the presence of the mutant suppressor tRNA and at lower temperatures (22°C or 30°C). The toxicity of the TyrRS gene was most likely caused by misaminoacylation of one of the *E. coli* tRNAs, with some compensation provided by the presence of another substrate, the suppressor tRNA, for the enzyme to act upon. The two major identity determinants for yeast TyrRS are the anticodon bases (shared only by the *E. coli* tRNA^{Tyr}, which is not a substrate for the yeast enzyme) and the C1:G72 base pair (12, 32, 33). This base pair is found only in tyrosine tRNAs of eukaryotes and archaeobacteria and proline tRNAs of *E. coli*. Therefore, we considered the possibility that yeast TyrRS was misaminoacylating *E. coli* tRNA^{Pro}. Aminoacylation reactions *in vitro* with purified yeast TyrRS and total *E. coli* tRNA demonstrated that the yeast enzyme is capable of aminoacylating tRNA^{Pro} with tyrosine (Figure 3). The toxicity of yeast TyrRS to *E. coli* appears at least in part dependent upon its intracellular concentration, as the gene was originally isolated by a functional selection, which relied on expression from a low copy yeast shuttle vector. However, for its use as a 21st synthetase, the yeast TyrRS first had to be modified so that it would no longer mischarge the *E. coli* tRNA^{Pro}.

***In vivo* selection for mutants of yeast TyrRS which are stable in *E. coli* and which can still aminoacylate the mutant tRNA₂^{fMet} (U2:A71/U35A36/G72).** The selection originally

used (20) for the isolation of the yeast TyrRS gene from a yeast cDNA library was adapted for the purpose of isolating TyrRS mutants, which could be stably maintained in *E. coli* (indicative of negligible mischarging of the endogenous tRNA^{Pro}), while requiring that the enzyme retain its aminoacylation activity with the mutant suppressor tRNA. The selection relied on co-expression of the suppressor tRNA, along with an amber mutant of the chloramphenicol acetyltransferase (CAT) gene, both carried by the pACamG plasmid. The activity of the yeast TyrRS was selected for by requiring growth on chloramphenicol, an indication that functional enzyme was present and was able to aminoacylate the suppressor tRNA. A library of yeast TyrRS mutants was prepared by error-prone PCR (Materials and Methods), cloned into the pKK223-3 plasmid, and used to transform *E. coli* CA274 carrying the pACamG plasmid. Twelve chloramphenicol resistant colonies were picked and the Su⁺ phenotype of three of them was confirmed further by restreaking on chloramphenicol plates (Figure 4A) and by screening for colony color on MacConkey lactose plates and scoring for growth on plates containing M9 minimal medium with lactose, which relied upon suppression of the two amber mutations in CA274 *lacZ(am)* and *trpA(am)* (data not shown). The stability of the three clones carrying the plasmids pKKTyrRS*5, pKKTyrRS*9, and pKKTyrRS*10 was confirmed by continued maintenance of the plasmids in liquid medium. Analysis of the *in vivo* aminoacylation levels of the mutant suppressor tRNA on acid-urea gels (28) showed that the tRNA was essentially quantitatively aminoacylated in the presence of any of the three yeast TyrRS mutants, in contrast to undetectable levels of aminoacylation in the absence of the enzyme (Figure 4B).

Sequencing revealed that three independent yeast TyrRS mutants had been isolated, with no overlapping mutations other than a serine to aspartate mutation found in the C-terminal myc-tags of TyrRS*5 and TyrRS*9. TyrRS*10 had a different mutation in the myc-tag, a glutamic

acid to valine change. TyrRS*10 had six additional mutations in the coding sequence, while TyrRS*5 had three and TyrRS*9 had only one (Table 1). All mutants also had several silent mutations, none of which appeared to involve poorly-utilized codons in *E. coli*. This latter result is consistent with the finding by immunoblot analysis that there were no appreciable differences in the levels of the various TyrRS mutants (data not shown). The mutations in TyrRS*10 were scattered throughout the gene, whereas the mutations in TyrRS*5 and TyrRS*9 were primarily in the C-terminal region of the protein, which is the binding domain for the anticodon region of the tRNA. None of the mutations fall in the connective polypeptide 1 (CP1) region, which has been shown to be important for discrimination of the base pair at position 1 and 72 in the tRNA (34).

***In vitro* characterization of the yeast TyrRS mutants.** The mutant TyrRS genes were cloned into the expression vector pET3d and the TyrRS mutants were purified. The enzymatic activity of the wild type, myc-tagged and the three mutant TyrRS proteins was determined with three substrates: yeast tRNA^{Tyr}, the mutant suppressor tRNA and *E. coli* tRNA^{Pro}. Interestingly, the mutations had very little effect on activity towards the natural substrate (yeast tRNA^{Tyr}) or the desired substrate (the suppressor tRNA) (Table 2). TyrRS*9, containing only one mutation, differed less than 2-fold in k_{cat}/K_m from the wild type with both substrates, whereas both TyrRS*5 and TyrRS*10 differed less than 3-fold. With the *E. coli* tRNA^{Pro} as substrate, however, there was a more significant effect, a decrease in k_{cat}/K_m from more than 5-fold (TyrRS*5) to as high as 10-fold (TyrRS*10). Some of the decrease in activity with this substrate appears to be a result of the addition of the C-terminal myc-tag, which was also seen to have a smaller effect on activity with the mutant suppressor tRNA. However, the myc-tag alone is unlikely to be responsible for the selection of these particular mutants, as only a limited number of chloramphenicol resistant colonies were isolated from the *in vivo* selection. Most importantly, all three of the mutants show

an increase in specificity for the suppressor tRNA over tRNA^{Pro} (Table 3), with TyrRS*9 preferring the suppressor tRNA over tRNA^{Pro} by a factor of 15-fold.

DISCUSSION

The 21st synthetase/tRNA pairs that we have developed for use in eukaryotes and in eubacteria consist, in both cases, of a heterologous aaRS and a suppressor tRNA derived from the initiator tRNA of the same organism or a related organism (12, 13). Besides providing the first example of a 21st synthetase/tRNA pair for use in a eukaryotic system, our approach differs somewhat from that of Schultz and coworkers, in which both the aaRS and the suppressor tRNA are imported from a heterologous organism (14, 15) and offers certain advantages. For example, because the suppressor tRNAs used are derived from tRNA of the same organism or a related organism, transcripts derived from the suppressor tRNA genes are likely to be processed well to yield functional suppressor tRNAs (35). Also, the suppressor tRNAs are likely to carry base modifications that are normally found in the tRNA and are, therefore, more likely to be optimally active in suppression (36). We have shown that the suppressor activity of the human initiator tRNA mutant in yeast is quite high. We have also compared the growth rates of *E. coli* CA274 *lacZ(am) trpA(am)* carrying either of the three mutant yeast TyrRS/suppressor tRNA pairs to that of an isogenic Su⁺ *E. coli* strain CA275, in a medium requiring suppression of the two amber alleles, and have found that the growth rates are similar (data not shown). Since the endogenous tyrosine suppressor tRNA in *E. coli* CA275 is a “strong” suppressor (37), the suppression efficiency of the 21st synthetase/tRNA pair must also be quite high. An important potential application of incorporation of amino acid analogues into proteins *in vivo* is the synthesis of large amounts of proteins carrying chemical or spectroscopic probes or heavy atoms such as

iodine at specific sites. This requires that suppression efficiency at the sites of incorporation of amino acid analogues be high. The systems that we have developed fulfill this requirement.

The approach used by Schultz and coworkers (14, 15) in which both the aaRS and the suppressor tRNA are imported from a heterologous organism, is based on the inability of a few eubacterial aaRSs to aminoacylate the corresponding tRNAs from eukaryotes or archaebacteria and *vice versa*. For example, the *E. coli* GlnRS does not aminoacylate yeast tRNA^{Gln} and the yeast GlnRS does not aminoacylate *E. coli* tRNA^{Gln} (38). Therefore, the import of such synthetase/tRNA pairs from a eukaryotic organism to eubacteria or *vice versa* would, at the outset, appear to provide a simple solution to the identification of potential 21st synthetase/tRNA pairs. However, although the GlnRS/tRNA^{Gln} suppressor combination appears to be portable in the case of both eukaryotes (35) and eubacteria (14), this cannot be used as a general approach. For example, the fact that yeast tRNA^{Tyr} is not aminoacylated by *E. coli* TyrRS does not mean that the suppressor derived from it will not be aminoacylated by other *E. coli* aaRSs. Initially, we considered the possibility of importing the yeast TyrRS and the suppressor derived from yeast tRNA^{Tyr} as the 21st synthetase/tRNA pair for use in *E. coli*. We found, however, that when expressed to moderately high levels, this tRNA was a good suppressor on its own in *E. coli* suggesting that it was aminoacylated by one of the *E. coli* aaRSs (Chow and RajBhandary, unpublished observations). This result agrees with a recent report of Wang *et al.* (15) but differs from that of Ohno *et al.* (39). Furter has also reported that an amber suppressor derived from yeast tRNA^{Phe} is aminoacylated in *E. coli* by LysRS (40).

Similarly, as shown here, the fact that the yeast TyrRS does not aminoacylate the *E. coli* tyrosine tRNA does not mean that it will not aminoacylate any of the other *E. coli* tRNAs. For example, although we initially cloned the gene for yeast TyrRS in *E. coli* on the basis of its

ability to complement the aminoacylation defect of a suppressor tRNA derived from the *E. coli* tRNA₂^{fMet} (20), we could not isolate transformants expressing the yeast TyrRS at moderate levels, presumably because the yeast TyrRS misaminoacylated *E. coli* tRNA^{Pro} with tyrosine (Figure 3) and was, therefore, toxic to *E. coli*. It was necessary to isolate mutants of yeast TyrRS which could be stably expressed in *E. coli*, most likely because of their much reduced activity in misaminoacylation of tRNA^{Pro}. The usual approach to circumvent the problem of toxicity of proteins being expressed in *E. coli* is to tightly regulate the expression of the protein. Such an approach is, however, not useful here because, once induced, the yeast TyrRS will still misaminoacylate the *E. coli* tRNA^{Pro} with tyrosine leading to misincorporation of tyrosine for proline in proteins. A further problem with the regulation of expression of the 21st synthetases, which have the capability of misaminoacylating endogenous tRNAs, would be the loss of site specificity of incorporation of the amino acid analogue in the target protein.

Kinetic analysis of the three yeast TyrRS mutants using the yeast tRNA^{Tyr}, the suppressor tRNA derived from *E. coli* tRNA₂^{fMet} and *E. coli* tRNA^{Pro} as substrates, shows that compared to the wild type yeast TyrRS, which prefers the suppressor tRNA over tRNA^{Pro} by a factor of about 2, with the mutant enzymes the corresponding numbers are between 5 and 15 (Table 3). Thus, relatively small decreases in activity of yeast TyrRS towards *E. coli* tRNA^{Pro} are enough to prevent the misaminoacylation *in vivo* of tRNA^{Pro} with tyrosine by yeast TyrRS. This is consistent with data showing that the high fidelity of aminoacylation of tRNAs *in vivo* is a result not only of positive and negative interactions of aaRSs with cognate and noncognate tRNAs, respectively, but is also achieved by maintaining a delicate balance of levels of enzymes and tRNAs which helps to prevent misaminoacylation reactions (41-43).

Finally, having fulfilled the requirement for two 21st synthetase/tRNA pairs, the next step is isolation of mutants in the 21st synthetases, which aminoacylate the suppressor tRNA with the amino acid analogues of choice instead of with the normal amino acid. This is likely to be a challenging step, possibly requiring multiple round of mutagenesis and selection to obtain a 21st synthetase, which shows an increase in selectivity for the amino acid analogue over the normal amino acid. Among the possible selection schemes are the use of (i) phage display of aaRS mutants (44) with selection based on binding to an affinity matrix consisting of an aminoalkyl-adenylate derived from the amino acid analogue and (ii) antibodies directed against peptides containing the amino acid analogue in a reporter protein expressed on the cell surface (11, 45) to select for cells carrying the desired aaRS mutant.

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LEGENDS TO FIGURES

Figure 1. Cloverleaf structure of the human initiator tRNA (left) and *E. coli* initiator tRNA (right). Arrows indicate the sequence changes in these tRNAs for their use as part of the 21st synthetase/tRNA pairs in yeast (left) and *E. coli* (right).

Figure 2. The human initiator tRNA mutant is aminoacylated in yeast only in the presence of *E. coli* GlnRS and is active in suppression. (A) Acid-urea gel analysis of the mutant tRNA expressed in *S. cerevisiae* HEY301-129, grown in SRD (minus *E. coli* GlnRS) or SRG (plus *E. coli* GlnRS). (B) Test for suppression of the *met8-1* amber allele in *S. cerevisiae* HEY301-129. *URA*⁺*LEU*⁺ transformants carrying pScM.M2 (the mutant suppressor tRNA) and pESC-LEU (plus and minus the *E. coli* GlnRS gene) were streaked on a selective plate (SRG) lacking methionine.

Figure 3. Acid urea analysis of *E. coli* tRNA^{Pro} in total *E. coli* tRNA incubated *in vitro* with purified yeast TyrRS and tyrosine.

Figure 4. The *E. coli* initiator tRNA mutant is aminoacylated in *E. coli* only in the presence of the yeast TyrRS mutants and the aminoacyl-tRNA is active in suppression. (A) Acid-urea gel analysis of the mutant tRNA expressed in *E. coli* in the presence and absence of the TyrRS mutants. (B) Growth of *E. coli* CA274 transformed with pACamG (carrying the mutant suppressor tRNA gene and the *CATam27* gene), and pKK223-3 (plus and minus the mutant TyrRS genes) on a 2X YT plate containing 100 µg/mL chloramphenicol.

Table 1. Sequence changes found in yeast TyrRS mutants that are stably expressed in *E. coli*. Only sense mutations are listed. Mutations found in the myc-tag region are indicated by parentheses, with the first amino acid of the tag numbered as 1'.

Mutant	Amino Acid Changes		
TyrRS*5	His285	→	Tyr
	Phe286	→	Tyr
	Lys372	→	Met
	(Ser9'	→	Asp)
TyrRS*9	Asn344	→	His
	(Ser9'	→	Asp)
TyrRS*10	Thr6	→	Ser
	Val35	→	Leu
	Asn113	→	Asp
	Met218	→	Ile
	Lys381	→	Thr
	Glu391	→	Gly
	(Glu1'	→	Val)

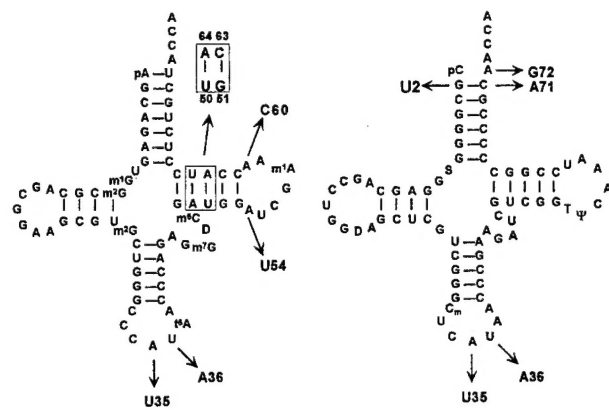
Table 2. Kinetic parameters of wild type and mutant yeast TyrRS.^a

tRNA substrate: Enzyme	k_{cat}/K_m ($s^{-1} M^{-1}$) $\times 10^6$	Relative k_{cat}/K_m
yeast tRNA ^{Tyr} :		
TyrRS (wt)	8.5	1
TyrRS-myc	11.8	1.2
TyrRS*5	3.1	0.36
TyrRS*9	6.7	0.79
TyrRS*10	3.6	0.54
suppressor tRNA:		
TyrRS (wt)	0.014	1
TyrRS-myc	0.0067	0.48
TyrRS*5	0.0077	0.55
TyrRS*9	0.015	1.1
TyrRS*10	0.0059	0.42
<i>E. coli</i> tRNA ^{Pro} :		
TyrRS (wt)	0.0061	1
TyrRS-myc	0.0016	0.26
TyrRS*5	0.0015	0.24
TyrRS*9	0.0010	0.16
TyrRS*10	0.00061	0.10

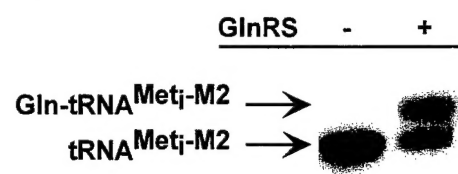
^a Kinetic parameters listed are the average of two or more experiments. The tRNA concentrations varied from 0.067 – 5X K_m . Enzyme concentrations ranged from 0.05 nM – 730 nM, depending on the tRNA used.

Table 3. Ratio of specificity constants for the aminoacylation of the U2:A71/U35A36/G72 mutant suppressor tRNA and *E. coli* tRNA^{Pro} by the wild type and mutant yeast TyrRS.

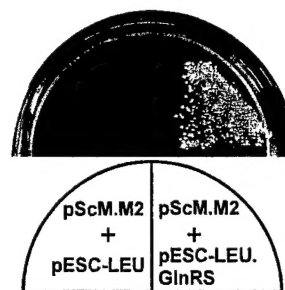
Enzyme	k_{cat}/K_m (suppressor tRNA)
	k_{cat}/K_m (tRNA ^{Pro})
TyrRS (wt)	2.30
TyrRS-myc	4.19
TyrRS*5	5.13
TyrRS*9	15.0
TyrRS*10	9.70



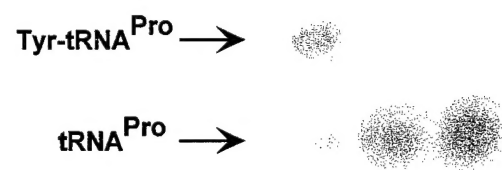
(A)



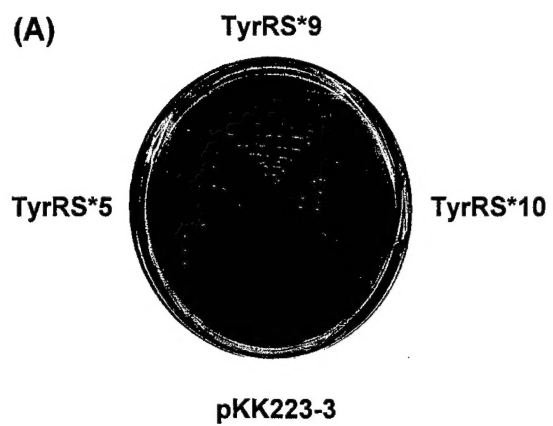
(B)



yeast TyrRS	+	+	-
tyrosine	+	-	-



(A)



(B)

pKK223-3	TyrRS*5		TyrRS*9		TyrRS*10		
-	-	+	+	+	+	+	aaRS
+	-	+	-	+	-	-	OH ⁻

